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Perspective

Liver macrophages in health and disease

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SUMMARY

Single-cell and spatial transcriptomic technologies have revealed an underappreciated heterogeneity of liver macrophages. This has led us to rethink the involvement of macrophages in liver homeostasis and disease. Identification of conserved gene signatures within these cells across species and diseases is enabling the correct identification of specific macrophage subsets and the generation of more specific tools to track and study the functions of these cells. Here, we discuss what is currently known about the definitions of these different macrophage populations, the markers that can be used to identify them, how they are wired within the liver, and their functional specializations in health and disease.

INTRODUCTION

Single-cell and spatial (proteo)genomics technologies have revolutionized our view on cellular heterogeneity. Within the liver, these technologies, including cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) and spatial transcriptomics, have allowed the heterogeneity of macrophages, their specific markers, and their unique locations to be assessed at unprecedented resolution. Although once considered to consist solely of a single population of resident Kupffer cells (KCs), we now know that both the murine and human liver harbor multiple populations of macrophages even in the steady state (Aizarani and Saviano, 2019; Andrews et al., 2022; Guilliams et al., 2022; MacParland et al., 2018), whereas additional populations are recruited in disease contexts (Guilliams et al., 2022; Krenkel et al., 2020; Ramachandran et al., 2019; Sharma et al., 2020). Moreover, spatial data enables the identification of the cells in the local environment of the macrophages, subsequently allowing the signals governing macrophage development to be defined (Bonnardel et al., 2019; Guilliams et al., 2020, 2022). Despite all the advantages of these technologies, there are also some limitations. One caveat is that many studies have defined distinct macrophage populations with their own unique nomenclature, despite there being considerable overlap across the different studies. Moreover, whether the distinct clusters of macrophages identified represent distinct populations or activation states is often unclear. Finally, these studies remain largely descriptive, limiting our understanding of cell functions. In this perspective, we aim to provide a detailed overview of the different hepatic macrophages in the murine and human liver in health and disease. Additionally, we will underline what is known about macrophage functions while also highlighting what we believe to be the next questions.

LIVER STRUCTURE AND CELLULAR COMPOSITION

The liver is responsible for a number of critical physiological processes, including metabolism, detoxification, and immunity (Trefts et al., 2017). The liver is organized into repeating hexagonal structural units termed lobules (Figure 1). Each central vein, which is connected to the hepatic vein and is responsible for returning deoxygenated blood to the heart, is surrounded by 6 portal triads. A portal triad consists of a portal vein that brings nutrient-rich blood from the intestines, spleen, and stomach to the liver (75% of hepatic blood), a hepatic artery which brings oxygenated blood from the heart to the liver (25% of the hepatic blood), and a bile duct, lined by specialized epithelial cells called cholangiocytes. All blood entering the liver then drains from the portal triads to the central vein via the liver sinusoids. Liver sinusoids are dynamic microvascular structures with fenestrations responsible for nutrient exchange between the blood and perisinusoidal space called the space of Disse (Gracia-Sancho et al., 2021; Trefts et al., 2017). Hepatocytes, the metabolic power houses of the liver, are found between the sinusoids, where they fulfill the functions of metabolism and detoxification depending on their location along the sinusoid (Halpern et al., 2017). Given the differences in the content of the blood at different distances from the central vein, these hepatocytes are metabolically zonated, which means that they exhibit functional heterogeneity based on their location within the lobule (Halpern et al., 2017). A similar zonation has also been reported in liver sinusoidal endothelial cells (LSECs) (Halpern et al., 2018). Stellate cells are mesenchymal cells found in the space of Disse that upon activation transdifferentiate into myofibroblasts, which deposit collagen, leading to fibrosis and cirrhosis (Trivedi et al., 2021). Kupffer cells (KCs) are the resident macrophages of the liver. KCs reside in the liver sinusoids, in prime position to sample and respond to any pathogens entering the

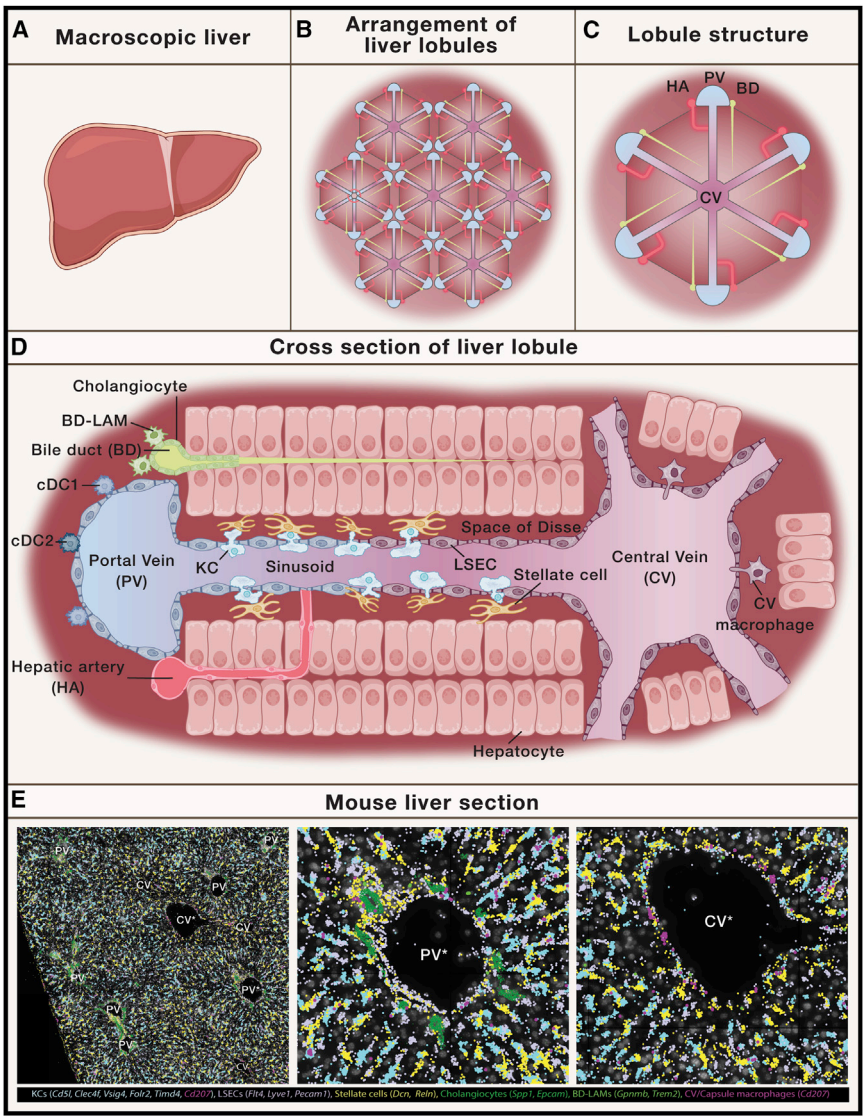

Figure 1. Hepatic macrophage subsets are anatomically distinct

(A–C) The liver is composed of repeating hexagonal lobules, whereby blood from the portal vein and hepatic artery drain through the sinusoids toward the central vein. Alongside the hepatic artery and portal vein is the bile duct, and these three structures combined comprise the portal triad.

(D and E) Macrophages are found throughout the liver structure as shown schematically (D) or by molecular cartography (mRNA fluorescence *in situ* hybridization, (see Guilliams et al., 2022) in the murine liver (E). Kupffer cells (KCs) are found in the liver sinusoids, in close contact with liver sinusoidal endothelial cells (LSECs). KCs project a proportion of their body in the space of Disse, where they are paired with a stellate cell. In addition to KCs, discrete populations of macrophages can be found at the bile duct, at the central vein and at the liver capsule. Those at the bile duct, called bile-duct lipid-associated macrophages (LAMs), express *Gpnmb* and *Trem2*, whereas those present at both the liver capsule and central vein express *Cd207* while lacking expression of other KC genes such as *Cd5l*, *Vsig4*, and *Timd4*.

liver via the blood. KCs also extend a considerable proportion of their body into the space of Disse, where they are in close contact with stellate cells and hepatocytes (Bonnardel et al., 2019). Alongside KCs, smaller populations of macrophages and dendritic cells (DCs) can also be found residing at the central vein and portal triads, as discussed below (Figure 1) (Guilliams et al., 2022; Sierro et al., 2017).

KUPFFER CELLS, THE RESIDENT MACROPHAGES OF THE LIVER

KCs were first described in 1876 by Wilhem von Kupffer and identified as macrophages 2 decades later by Tadeusz Browicz (Browicz, 1899; Kupffer, 1876; Wake, 2004). Despite being one of the oldest immune cells known to man, we still know relatively little about these cells, especially regarding their functions. This is because, until recently, it was not possible to discriminate KCs from other hepatic macrophages, which all express generic macrophage markers including CD64, F4/80, and MerTK. However, in recent years, considerable advances have been made in our ability to discriminate KCs. CLEC4F was identified as a specific marker of murine KCs, enabling the generation of *Clec4f-Dtr* and *Clec4f-cre* mouse models to specifically deplete or target KCs, respectively (Beattie et al., 2016; Sakai et al., 2019; Scott et al., 2016, 2018). Although CLEC4F is a useful KC marker, it also has its limitations. It is expressed relatively late in KC development, making it difficult to identify cells that are developing into KCs (Bonnardel et al., 2019; Remmerie et al., 2020a; Scott et al., 2016, 2018). Although not an issue in the healthy liver, where KCs are primarily of embryonic origin and self-maintain throughout life (Ginhoux and Guilliams, 2016; Ginhoux and Jung, 2014; Hoeffel et al., 2015; Liu et al., 2019; Schulz et al., 2012), this causes problems in the diseased liver, where KCs are replaced by monocyte-derived KCs (moKCs) (Blériot et al., 2015; Daemen et al., 2021; Remmerie et al., 2020a; Seidman et al., 2020; Tran et al., 2020). Here, moKCs can be present for almost a week before they can be discriminated from other hepatic macrophages as CLEC4F^{+} cells. Second, although conserved across many species, CLEC4F is not conserved in humans (Guilliams et al., 2022). This has contributed to the ongoing lack of a consensus regarding the identity of *bona fide* human KCs.

Studies profiling the cells of the healthy liver using single-cell or single-nucleus RNA sequencing (sc/snRNA-seq) have hoped to shed light on the true identity of human KCs. However, each study performed has reached different conclusions regarding human KC identity (Table 1). Many studies have suggested two populations of human KCs (Aizarani and Saviano, 2019; Andrews et al., 2022; MacParland et al., 2018; Ramachandran et al.,

Table 1. Human KC gene markers proposed by distinct sc/snRNA-seq studies

Guiilliams/Scott Cell 2022	Andrews/Macparland Hep. Comms 2021		Aizarani/Grun Nature 2019		Ramachandran/ Henderson Nature 2019		Sharma/Dasgupta Cell 2020		
KCs (conserved)	non-inflam Macs	inflam Macs	MARCO+ KCs	CD1C+ KCs	KC1	KC2	TAM1	TAM2	TAM3
CD5L	*VCAM1*	*VCAN*	*C1QC*	*FCER1A*	*NDST3*	*CD5L*	*CD5L*	*SPP1*	*MT1H*
VSIG4	*NDST3*	*FCN1*	*C1QA*	*CD1C*	*TIMD4*	*VCAM1*	*FOLR2*	*NUPR1*	*MT1G*
SLC1A3	*KIAA1217*	*MNDA*	*FOLR2*	*RNASE6*	*CD5L*	*CETP*	*SEPP1*	*RNASE1*	*MT1M*
CD163	*MARCO*	*S100A12*	*C1QB*	*HLA-DQB1*	*CETP*	*CXCL12*	*SLC40A1*	*PLTP*	*MT1E*
FOLR2	*MPPED2*	*AREG*	*S100A9*	*CPVL*	*VCAM1*	*SDC3*	*APOE*	*COLEC12*	*MT1X*
TIMD4	*LILRB5*	*LYZ*	*MARCO*	*FCGR2A*	*LYVE1*	*RND3*	*C1QC*	*SCD*	*MT1F*
MARCO	*SLC40A1*	*S100A8*	*MS4A7*	*HLA-DMB*	*MARCO*	*SLC40A1*	*MS4A4A*	*MMP19*	*APOC3*
GFRA2	*EXT1*	*AC020656.1*	*ADAP2*	*CLEC10A*	*SLC16A9*	*LYVE1*	*C1QB*	*CXCL3*	*ORM1*
ADRB1	*TPRG1*	*S100A6*	*CD163*	*CSF1R*	*SDC3*	*MMP19*	*LILRB5*	*CTSD*	*APOA2*
TMEM26	*FRMD4A*	*S100A4*	*SIGLEC1*	*VSIG4*	*CXCL12*	*LGMN*	*IGF1*	*MMP9*	*ALB*
SLC40A1	*DST*	*IL1R2*	*CD5L*	*CD86*	*LILRB5*	*MARCO*	*C1QA*	*CD109*	*IFI27*
HMOX1	*SLCO2B1*	*TSPO*	*CYBB*	*CD163*	*SCD*	*HMOX1*	*LGMN*	*SLC16A10*	*PSMB3*
SLC16A9	–	*S100A9*	*HMOX1*	*CLEC7A*	*SLC40A1*	*CD163*	*CCL18*	*CSTB*	*MINOS1*
VCAM1	–	*RNASE2*	*CFD*	*CD83*	*SELENBP1*	*SEPP1*	*VCAM1*	*CCL2*	*COPE*
SUCNR1	–	*CSTA*	*S100A8*	*AIF1*	*C2*	*CTSL*	*A2M*	*CTSZ*	*RBX1*
–	–	*CAPG*	*AIF1*	*HLA-DOA*	*FABP3*	–	*GPR34*	*ABL2*	*PSMA4*
–	–	*LGALS1*	*CSF1R*	*IL1B*	*SEPP1*	–	*LIPA*	*MMP14*	*ATP5G3*
–	–	*CORO1A*	*LILRB5*	*GPR183*	*TMEM37*	–	*GPNMB*	*PLIN2*	*ARPC5*
–	–	*CD37*	*MS4A4A*	*HLA-DMA*	*FOLR2*	–	*C2*	*FN1*	*HN1*
–	–	*S100A10*	*C5AR1*	*HLA-DQA1*	*RND3*	–	*DAB2*	*CTSB*	*PRDX5*

2019), although the evidence that these are *bona fide* KC populations is still lacking. By comparing human and mouse, we have recently suggested a core gene signature specific to KCs (Table 1), including *CD5L*, *VSIG4*, *CD163*, *FOLR2*, *MARCO*, and *SLC40A1*, that can be used to identify a single homogeneous population of KCs within the liver across 7 different species (Guilliams et al., 2022). Examination of this "KC signature" across the different existing sc/snRNA-seq datasets consistently identified a population of cells, suggesting that this may indeed be a useful signature to identify KCs (Figure 2). In most cases, this only identified one of the subpopulations of the cells originally annotated as KCs, suggesting that the reported KC heterogeneity observed in these studies may be due to the contamination of the genuine KCs with other myeloid populations (Figure 2). The lack of consensus regarding KC identity can be further demonstrated by mapping the top differentially expressed genes (DEGs) originally proposed for the different "KC" subsets across the datasets. This reveals that many of these additional "KC" subsets have been annotated as monocytes, cDCs, or non-KC macrophages in other studies (Figure 2). Although a consensus still needs to be reached by the community on the exact identity of the different hepatic myeloid cells, for human KCs, we would propose that a gene list defined by comparison of liver cells across species may be useful (Guilliams et al., 2022). Much of this KC "signature" is not restricted to gene expression and, thus, can also be used to identify KCs across species by flow cytometry (based on protein), including VSIG4, FOLR2, and CD163 (Figure 2). In addition, other surface receptors on murine KCs have also been identified, further enabling their discrimination from other hepatic macrophages, including CLEC2 and CD206 (Figure 2). CLEC2 (encoded by *Clec1b*) is a very early marker of KCs that remains expressed throughout their lifespan (Remmerie et al., 2020a; Tran et al., 2020); thus, this is a very useful marker to identify moKCs before they express CLEC4F or VSIG4. On the other hand, TIM4 and CD163 are acquired later in moKC development, allowing them to be utilized to distinguish recently derived moKCs from those that have resided in the liver for some time (Table 2) (Beattie et al., 2016; Scott et al., 2016). Although moKCs in mice are largely restricted to the diseased liver (Daemen et al., 2021; Remmerie et al., 2020a; Seidman et al., 2020; Tran et al., 2020) or following KC depletion (Beattie et al., 2016; Bonnardel et al., 2019; Sakai et al., 2019; Scott et al., 2016), in the healthy human liver, such cells have been identified in sc/snRNA-seq datasets (Guilliams et al., 2022), possibly because humans are exposed to pathogens, fatty diets, alcohol, and other toxins that may lead to moKC recruitment (see below). However, whether these are genuine moKCs remains to be investigated. Studies of HLA-mismatched transplanted livers have identified host- and donor-derived macrophages in the transplanted livers, suggesting that moKCs may indeed be generated in the healthy human liver (Pallett et al., 2020). However, whether these represent genuine moKCs is also unclear. The only KC-specific marker used in this study was CD163, and its expression was lower in host-derived macrophages compared with donor macrophages (Pallett et al., 2020). In mice, *Cd163* is one of only a handful of DEGs between moKCs and resident KCs (Scott et al., 2016), but additional work is still needed to validate this nomenclature in the human liver.

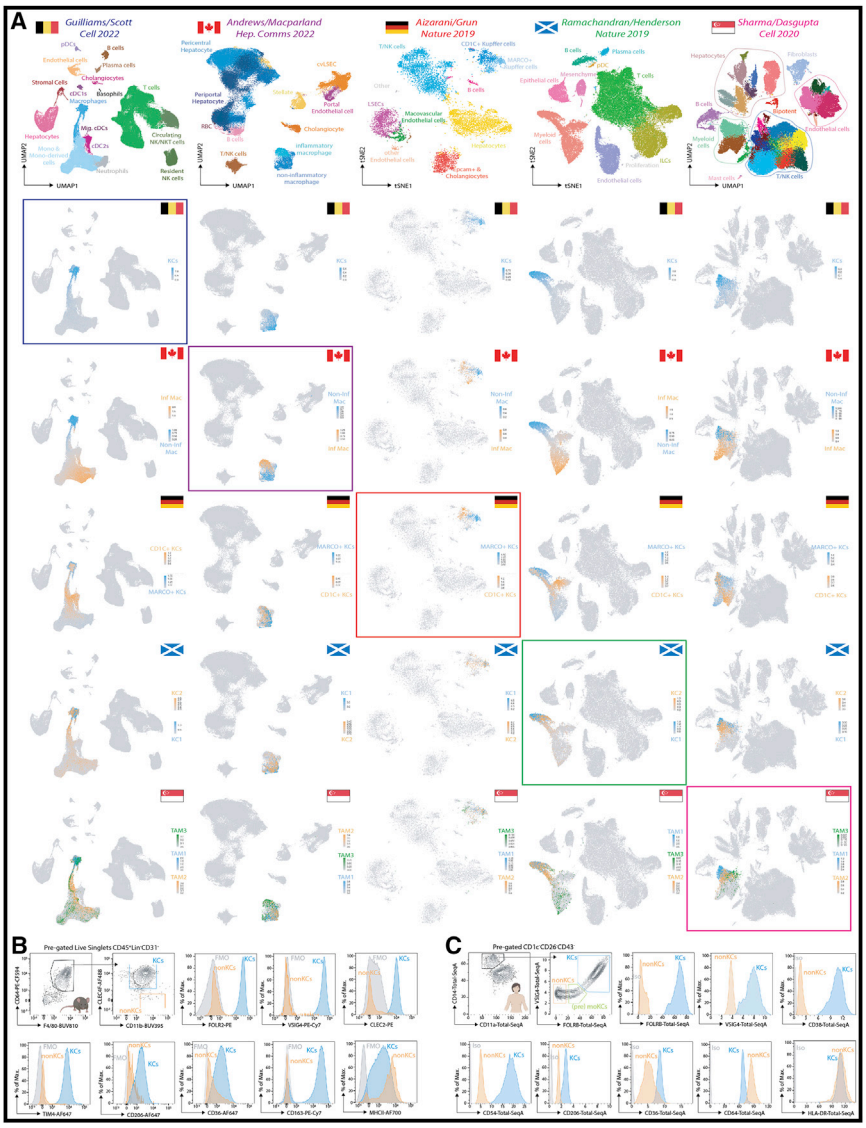

Figure 2. Identification and alignment of human KCs across health and disease

(A) To identify and align the *bona fide* KC populations across studies, data from 5 recent human liver cell atlas papers were downloaded and Uniform Manifold Approximation and Projections (UMAPs) or t-distributed stochastic neighbor embedding (tSNE) plots from the studies recreated and annotated as originally published (Aizarani and Saviano, 2019; Andrews et al., 2022; Guilliams et al., 2022; Ramachandran et al., 2019; Sharma et al., 2020). In each of the studies, signatures were obtained for the different cell types annotated as KCs or macrophages by examining the differentially expressed genes (DEGs) in these populations compared with the other myeloid cells annotated within each study (Table 1). These signatures were then plotted on each of the UMAP or tSNE plots to examine where the cells would map in the other studies.

(B and C) Representative histograms showing expression of KC-specific markers in (B) mouse liver by flow cytometry or (C) human liver by CITE-seq by KCs and non-KC macrophages compared with either fluorescence minus one (FMO) (flow cytometry) or isotype (CITE-seq) controls. CD163 only stains a fraction of KCs when stained extracellularly, but this fraction is increased when stained intracellularly. Data were obtained from Guilliams et al. (2022).

OTHER MACROPHAGE POPULATIONS IN THE HEALTHY LIVER

In addition to KCs, additional macrophage populations have been described in the liver (Figure 1). First, a population of liver capsule macrophages (LCMs) have been identified (Sierro et al., 2017). The liver capsule consists of a single layer of mesothelial cells and a single stratum of capsular fibroblasts existing just beneath the surface of the liver (Balog et al., 2020). LCMs exist within this matrix in both the murine and human liver (Guilliams et al., 2022; Sierro et al., 2017). In the murine liver, LCMs derive from monocytes and accumulate in the capsule at weaning (Sierro et al., 2017). They express general macrophage markers, such as F4/80 and CD64, but do not express KC markers, such as VSIG4, CLEC4F, FOLR2, or CLEC2 (Guilliams et al., 2022). Rather they express CX3CR1 and CD207 (Guilliams et al., 2022; Sierro et al., 2017), although CD207 is also expressed by KCs (Guilliams et al., 2022), further highlighting the need to utilize KC-specific markers to exclude KCs from any analysis of LCMs (Table 2). Although LCMs have a defined signature in mice, a signature for these cells in humans remains elusive. This may be a result of a lack of conservation between mice and humans (Guilliams et al., 2022) but also likely reflects the lack of capsule tissue in human liver biopsies.

Although LCMs have been relatively well characterized in the murine liver, their transcriptional signature is not unique among hepatic macrophages, as it is also shared with a population of macrophages present at the central vein (Guilliams et al., 2022). These macrophages are, however, distinct from *Xcr1*$^{+}$*Flt3*$^{+}$

There is also an ongoing debate regarding KC homogeneity in the healthy murine liver (Hume et al., 2022; Iannacone et al., 2022). It has recently been proposed that KCs could be split into two populations on the basis of expression of CD206 and ESAM, with CD206^{-}ESAM^{-} KC1s being the majority population alongside a smaller population of CD206^{+}ESAM^{+} KC2s (Blériot et al., 2021; Simone et al., 2021). Although comparison of the transcriptomes of KC1s and KC2s reveals a number of DEGs between these two populations, all of the genes specifically found in KC2s are also expressed by LSECs, raising the possibility that these cells may be doublets (Guilliams et al., 2022), or that this may be a digestion artifact (Hume et al., 2022). Taken together, although further research is required to reach a consensus on KC homogeneity in the murine and human liver, through the utilization of a combination of KC-specific surface markers, including VSIG4, CLEC4F, and FOLR2, and genes including *CD5L*, *SLC40A1*, *MARCO*, and *CD163*, we can finally discriminate KCs from other macrophages.

Table 2. Protein and gene markers delineating hepatic macrophage subsets in the healthy and diseased liver

KCs	moKCs	LAM-like KCs[c]	LAMs	LCMs & CV macrophages[c]
VSIG4	VSIG4	VSIG4	*GPNMB*	CD207
FOLR2	FOLR2	FOLR2	*SPP1*	CX3CR1
CD163	CLEC4F	CD163	*CD9*	*Ccr2*
CLEC4F[a]	CLEC2	CLEC4F	*TREM2*	–
CLEC2[a]	*CD5L*	CLEC2	CD63	–
TIM4[d]	*MARCO*	TIM4	*CCR2*	–
CD5L	*GFRA2*	*CD5L*	CD141[b]	–
MARCO	*ADRB1*	*GPNMB*	CD26[b]	–
GFRA2	*TMEM26*	*SPP1*	*CX3CR1*	–
ADRB1	*SLC40A1*	*MMP12*	*FABP5*	–
TMEM26	*HMOX1*	*CD9*	*CLEC4D*	–
SLC40A1	*SLC16A9*	*TREM2*	*CLEC4E*	–
HMOX1	*VCAM1*	CD207	–	–
SLC16A9	*SUCNR1*[b]	–	–	–
VCAM1	*CD207*	–	–	–
SUCNR1[b]	–	–	–	–
CD207	–	–	–	–

[a]Only expressed in mouse.
[b]Only expressed in human.
[c]Population only described to date in mouse.
[d]Gene expressed in human and mouse, but protein only detected to date in mouse.

conventional type I DCs (cDC1s), which are also found around the veins and express CD207 (Guilliams et al., 2022). Alongside these LCMs and central vein macrophages, we also described an additional population of macrophages in the healthy murine and human liver in close proximity to the bile ducts (Guilliams et al., 2022) (Figure 1). These macrophages share their transcriptional profile across species and resemble macrophages described in obese adipose tissue and the fatty liver, termed lipid-associated macrophages (LAMs) (Jaitin et al., 2019; Remmerie et al., 2020a), expressing genes including *Gpnmb*, *Spp1*, *Trem2*, and *Cd9* (Table 2). This led us to refer to these cells as bile-duct LAMs (BD-LAMs) (Guilliams et al., 2022) (Box 1). Unfortunately, we still rely on negative gating strategies for BD-LAMs in the murine liver (CLEC4F$^-$VSIG4$^-$CLEC2$^-$CD207$^-$CD64$^+$F4/80$^+$), as none of these genes have proven useful as surface markers for these cells. However, in the human liver, BD-LAMs are identifiable in CITE-seq analysis based on their expression of CD14, CD11a, CD26, CD141, and CD9 (Table 2) (Guilliams et al., 2022). From these studies, it is clear that although KCs constitute the major macrophage population in the healthy liver, they exist alongside at least two other macrophage populations.

THE INTEGRATED NICHE: WIRING KC FUNCTION THROUGH INTEGRATED AND INTERCELLULAR CIRCUITS

In recent years, we have gained some insight into KC functions. Alongside their stereotypical roles as phagocytes in tissue homeostasis and immune surveillance, KCs also express many genes that are not involved in immune defense or expressed by other tissue-resident macrophages (Scott et al., 2018), suggesting that they may fulfill liver-specific functions. Parenchymal cells do not perform tissue-specific functions autonomously but rather have outsourced important tasks to other resident cells, including macrophages (Guilliams and Scott, 2017; Okabe and Medzhitov, 2016). For example, the liver is the main organ responsible for iron recycling in vertebrates (Michels et al., 2015), which is crucial to avoid iron deficiency due to the limited amounts of nutritional iron (Pasricha et al., 2021). Therefore, iron bound to heme in hemoglobin from aged red blood cells (RBCs) needs to be taken up, processed, stored, and then shuttled back to the bone marrow, where it is provided to new RBCs. The liver is the main site of both uptake of aged RBCs and iron storage (Slusarczyk and Mleczko-Sanecka, 2021; Theurl et al., 2016), but how is this achieved? First, aged or damaged RBCs need to be recognized and scavenged, which occurs through specific receptors including TIM4 (*TIMD4*), AXL, and CD36 (Slusarczyk and Mleczko-Sanecka, 2021). Iron within heme molecules needs to be degraded by Heme-oxygenase-1 (*HMOX1*), but, as HMOX1 is in the cytoplasm, heme first needs to be exported from the lysosome to the cytoplasm via HRG1 (*SLC48A1*) (White et al., 2013). Free hemoglobin in circulation is toxic for many organs and needs to be recycled, which occurs by association with haptoglobin that is taken up by cells expressing the scavenger receptor CD163 (Slusarczyk and Mleczko-Sanecka, 2021). The iron obtained from these processes then needs to be exported out of the cells by Ferroportin (*SLC40A1*) (Zhang et al., 2011). Most cells acquire iron from circulating transferrin (*TF*), the main iron transporter (Michels et al., 2015; Pasricha et al., 2021). Overall, systemic iron homeostasis is primarily controlled by hepcidin (*HAMP*), which binds to Ferroportin blocking cellular iron export (Michels et al., 2015; Nemeth et al., 2004a). Another regulator of iron homeostasis is HFE (homeostatic iron regulator) in which mutations cause hereditary hemochromatosis (Makui et al., 2005). The expression of hepcidin is further controlled by the bone morphogenic protein-6 (BMP6)-BMP-receptor-SMAD pathway (Mayeur et al., 2014) and the interleukin-6 (IL-6)-IL-6-receptor-STAT3 transcription factor pathway (Nemeth et al., 2004b). Finally, iron can also be stored intracellularly when coupled to ferritin (Anderson and Shah, 2013; Kaplan and Ward, 2013).

Hepatocytes represent the main cellular storage place of iron in the body and express high amounts of ferritin (Anderson and Shah, 2013). They also express many other genes involved in iron metabolism (Figure 3). However, alone they are not sufficient to carry out this process in its entirety. Genes involved in uptake of RBCs and haptoglobin, the export of heme to the cytoplasm, degradation of heme, export of iron outside the cell, and regulation of hepcidin expression are primarily expressed by KCs or LSECs (Figure 3). Importantly, KC expression of many of these genes including *SLC40A1*, *HMOX1*, *HRG1*, *HFE*, *TIM4*, *AXL*, and *CD163* is strongly conserved across species (Guilliams et al., 2022) (see www.livercellatlas.org), whereas mutations in *HFE*, *HRG1*, *SLC40A1*, and *HMOX1* are all linked to hereditary forms of anemia (Camaschella and Pagani, 2021). Similarly, mice lacking *Slc40a1* in macrophages also develop anemia and show hepatic iron accumulation (Zhang et al., 2011), whereas anemia in *Hmox1*-deficient mice could be rescued by

Box 1. Hepatic macrophages—What's in a name?

The precise name given to a population of macrophages influences the way we interpret scientific data. As many macrophages are recruited to the inflamed liver, we can no longer call any macrophage in the liver a KC. Calling all these cells KCs will wrongly imply that KCs are plastic. We, therefore, need to encourage the use of KC-specific markers to be able to discriminate KCs from other hepatic macrophages (Table 2). In mice, these KC-specific markers include CLEC4F, VSIG4, CLEC2, and FOLR2 (Figure 2) (Guilliams et al., 2022). KC-specific mouse models have been constructed based on CLEC4F expression (Sakai et al., 2019; Scott et al., 2016, 2018). Of course, KC fate-mapping will be required to ascertain that KCs do not lose the expression of these surface markers during disease. In humans, no consensus has been reached yet, but the alignment performed between the different single-cell sequencing studies of the human liver (Figure 2) and the comparison with the single-cell liver data from other species (Guilliams et al., 2022) point toward a strongly conserved KC signature (Table 2). VSIG4 and FOLR2 also appear to be robust human KC protein markers (Figure 2). A conserved signature is also found for LAMs. Given the big overlap between LAMs identified in the murine fatty liver and in the obese adipose tissue (Jaitin et al., 2019), we opted to use this name to maintain continuity across studies. However, is lipid-associated macrophage the most appropriate name for these cells? KCs also express many genes associated with lipid processing (Guilliams et al., 2022) and their neutral lipid content is considerably higher than that of LAMs in both the steady-state and the fatty liver (Remmerie et al., 2020a). Macrophages with a very similar gene signature have also been given other names such as scar-associated macrophages (SAMs) in the cirrhotic liver or NASH-associated macrophages (NAMs) in the fatty liver (Ramachandran et al., 2019; Xiong et al., 2019). However, as these cells are found in the steady-state liver around the bile-duct and across distinct disease models, both in the presence and absence of fibrotic scars, neither of these names seem appropriate. For the moment, we would propose to continue to call these cells LAMs until we understand the common signals that drive the development of these cells in the different models, so that we can adapt the nomenclature to include the factors regulating their induction.

transferring wild-type macrophages that homed to the liver (Kim et al., 2018). Throughout evolution, hepatic iron homeostasis has, therefore, evolved to be an intercellular collaborative process between hepatocytes, KCs and LSECs.

A similar analysis can be done for cholesterol homeostasis. Although hepatocytes are crucial for this process (Chiang and Ferrell, 2018; Luo et al., 2020; Wulp et al., 2013), hepatocytes alone are not sufficient. Indeed, KCs express many receptors involved in the removal of oxidized low-density lipoprotein (ox-LDL) from circulation, including SR-A1 (*MSR1*), CD36, TIM4 (*TIMD4*), and AXL. This is thought to protect from disease as increased concentrations of ox-LDL are associated with exacerbated cardiovascular disease (Ehara et al., 2001; Hulthe and Fagerberg, 2002). These receptors also recognize dying cells, and this helps to recycle the cholesterol in cell membranes. The LDL and membrane lipids from apoptotic cells are then degraded to free fatty acids and cholesterol by the lysosomal acid lipase (*LIPA*) and exported to the cytosol by NPC1 and NPC2. The cholesterol is subsequently exported from the KCs through ABCA1 and ABCG1 and loaded on nascent high-density lipoprotein (HDL) to form mature HDL (Figure 3). Again, this function of KCs is highly conserved across multiple species (Guilliams et al., 2022) and is crucial for homeostasis. Mice lacking *Abca1* and *Abcg1* in myeloid cells develop exacerbated atherosclerosis (Westerterp et al., 2013). LIPA-deficient mice show massive accumulation of lipids in the liver (Du et al., 1998) and humans carrying *LIPA* mutations have lower amounts of circulating HDL and an increased risk of cardiovascular disease (Wild et al., 2011). Human, but not murine KCs, also specifically express the cholesteryl ester transfer protein (*CETP*). CETP is involved in the transfer of cholesteryl esters from HDL to LDL (Figure 3) and humans carrying CETP mutations have higher HDL (Inazu et al., 1990). Moreover, expression of human CETP and human APOE in mice induces a shift in lipoprotein composition and induces HDL and LDL concentrations similar to those found in humans (Grass et al., 1995). Interestingly, human CETP is mainly expressed in KCs in these transgenic mice, demonstrating a conserved regulation of gene expression (Tuin et al., 2018).

What are the regulatory mechanisms that induce the expression of these cholesterol and iron homeostasis genes in KCs? LXRα (*NR1H3*) and SPIC are two transcription factors involved in the expression of much of the lipid- and iron-handling machinery of macrophages (Haldar et al., 2014; Kohyama et al., 2009; Sakai et al., 2019; Scott et al., 2018). LXRα is a master transcription factor of KCs, controlling the cholesterol-processing machinery but also the expression of many immune related factors including IL-18bp, complement molecules (C1 and C6), and the chemokine CXCL13 (Sakai et al., 2019; Scott et al., 2018). The transcription factor ID3 has also been proposed to control KC identity (Mass et al., 2016). Importantly, it is not just the functions of the different cells that are integrated, the liver signals that induce the expression of *NR1H3*, *SPIC*, and *ID3* in KCs also reveal an integrated intercellular circuitry within the niche in which different signals synergize together (Figure 3): DLL4 from LSECs, BMP9 and 10 from stellate cells, and unknown factors from hepatocytes (Bonnardel et al., 2019; Guilliams et al., 2022; Mass et al., 2016; Sakai et al., 2019; Zhao et al., 2022). BMP9-ALK1 stimulation alone does not induce *Nr1h3* in monocytes but requires synergy with DLL4-NOTCH2 (Bonnardel et al., 2019). This is because the SMAD-transcription factors induced by the transforming growth factor (TGF)-BMP signaling pathways require collaboration with co-factors for efficient DNA binding and transcription (Mullen et al., 2011; Trompouki et al., 2011). DLL4-Notch2 signaling induced by the transcription factor RBPj seems to be one of the factors required to potentiate BMP9-ALK1-SMAD signals in KCs (Bennett et al., 2020). Consistent with this, KCs are only found in the peri-portal region of the liver where LSECs produce DLL4 and not in the peri-central zone where LSECs do not express Notch ligands (Guilliams et al., 2022). Co-culture of hepatocytes with monocytes induces *Id3* expression (Bonnardel et al., 2019), whereas loss of ALK1 decreases it (Zhao et al., 2022). Yet, *in vitro* stimulation of monocytes with BMP9 alone

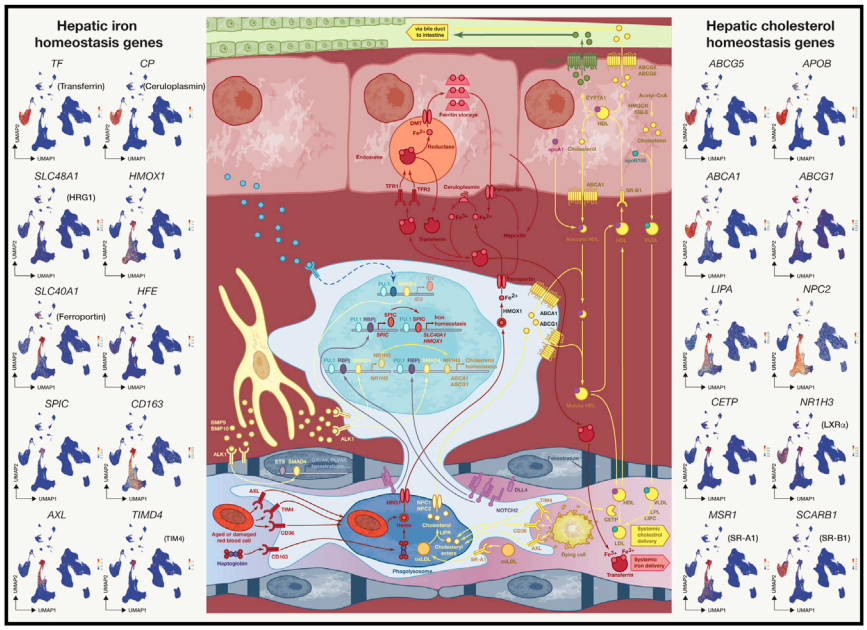

Figure 3. Integrated KC function in hepatic iron and cholesterol homeostasis

Schematic representation of iron and cholesterol homeostasis pathways showing the contribution of hepatocytes, LSECs, stellate cells, and KCs. UMAPs show the expression of genes involved in iron and cholesterol homeostasis in the human liver (Guilliams et al., 2022) to illustrate that some key genes are only expressed by specific cells, suggesting that hepatocytes, LSECs, stellate cells, and KCs function together as an integrated niche. See Figure 2A for the annotation of the cell types in the UMAPs.

does not yield high *Id3* expression. We, therefore, speculate that induction of *ID3* in KCs requires an additional hepatocyte-induced co-factor for the SMAD complex (Figure 3).

The crucial role of the BMP9-ALK1-SMAD pathway in KCs is demonstrated in ALK1-deficient monocytes that fail to develop into KCs, whereas loss of ALK1 from KCs leads to a loss of identity and death (Guilliams et al., 2022; Zhao et al., 2022). The BMP9-ALK1 interaction also controls the GATA4-transcription-factor-driven liver-specific identity of LSECs, inducing the expression of the membrane protein PLVAP that plays an essential role in the generation of LSEC fenestrations (Desroches-Castan et al., 2019). These cell-cell interactions are highly conserved across species (Guilliams et al., 2022) and humans carrying ALK1 mutations develop hereditary hemorrhagic telangiectasia, which is associated with severe vascular malformations in the liver, necessitating liver transplantation (Lerut et al., 2006). The WNT signals produced by peri-central LSECs are also conserved across species and are essential to control the peri-central function of hepatocytes (Hu et al., 2022). Altogether, this suggests that throughout evolution hepatocytes, LSECs, stellate cells, and KCs have become genetically integrated to function together as one intercellular functional module, highlighting that these cells represent one another's niche. This broadens the concept of the macrophage niche beyond the local supply of growth factors, such as CSF1. We would, therefore, like to propose a more modular view in which the liver sinusoids are constituted of repeated 4-cell building blocks consisting of a KC, a stellate cell, an LSEC, and a hepatocyte. To underline that the functional identity of each cell is controlled by integrated cell-cell circuits, we propose the term "integrated niche."

A strongly intertwined wiring of the transcriptomic identity would ensure that the division of labor between the 4 cells constituting the integrated niche is correctly imprinted and coordinated, but this would also restrict the plasticity of the members of the integrated niche. Originally, it was proposed that macrophages would require local CSF1-receptor ligands to develop and self-maintain in tissues, but that tissue-specific functions would then be imprinted in each individual resident macrophage by plastic adaption to the functional needs of each tissue through exposure to local nutrients, such as retinoic acid (inducing GATA6 in the peritoneum), heme (inducing SPIC in the liver and spleen macrophages), or surfactant (inducing PPARγ in alveolar macrophages) (Okabe and Medzhitov, 2016; Zhou et al., 2018). Strong genetic integration of macrophages into pre-defined tissue programs was dismissed (Okabe and Medzhitov, 2016), because it would not allow the alleged plasticity of macrophages (Locati et al., 2020; Wynn et al., 2013). However, single-cell transcriptomics and fate-mapping studies have questioned the widely assumed extreme plasticity of tissue-resident macrophages (Guilliams and Svedberg, 2021). Moreover KCs, peritoneal macrophages, or intestinal macrophages could not adapt to the lung environment when transferred in the alveolar space (van de Laar et al., 2016). Conversely, we could induce *Spic* and *Nr1h3* expression in monocytes *in vitro* solely by exposing the cells to DLL4 and BMP9 (mimicking LSEC and stellate cell wiring) (Bonnardel et al., 2019). This does not exclude a role for phagocytosis in shaping macrophage identity but suggests that their gene expression is for a big part controlled by cell-cell interactions within the integrated niche. The strong interconnected character of the integrated niche has important consequences for the development of liver organoids, as the genetic integration of the different regulatory programs implies that one will have to build organoids containing hepatocytes, LSECs, stellate cells, and KCs before each of these cells will acquire their *in vivo* functionalities. Finally, it has important consequences for our understanding of liver diseases, as prolonged injury may disrupt the integrated niche, which in turn may destabilize the whole hepatic ecosystem, as discussed below.

KUPFFER CELLS IN THE DISEASED LIVER

A common feature across liver diseases is the reduction or loss of the resident KC population (Figure 4). This has been observed in numerous murine liver disease models including non-alcoholic fatty liver disease (NAFLD) and non-alcoholic

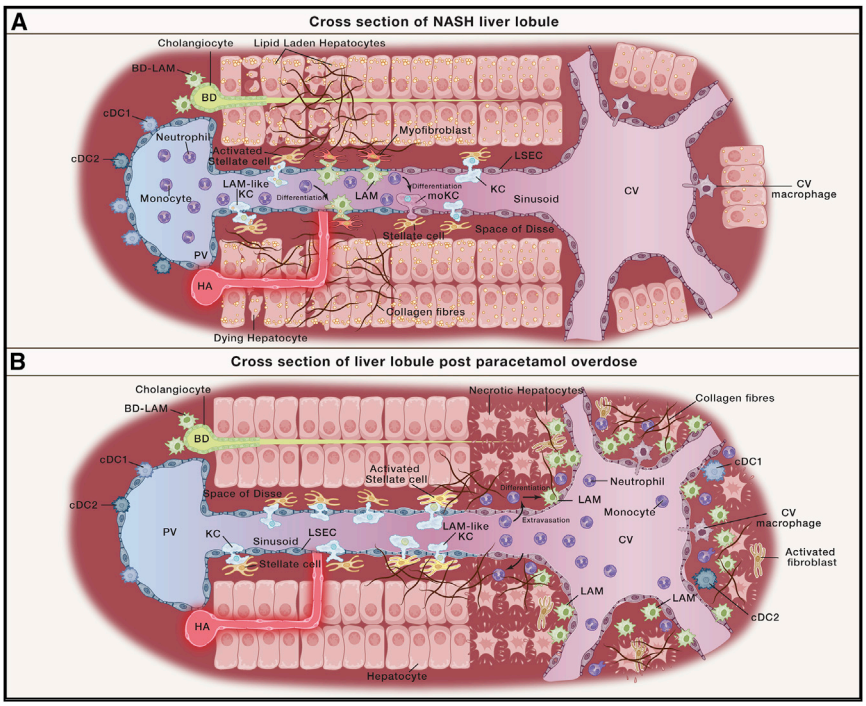

Figure 4. LAM and LAM-like KC development in the inflamed liver is restricted to sites of tissue damage

(A and B) In NASH and paracetamol overdose (APAP), two very distinct models of hepatic disease, monocytes have been shown to home to the specific zones of damage and to differentiate into LAMs.

(A) In NASH, these cells replace dying KCs in zones of steatosis and fibrosis, whereas the few KCs lost from the less steatotic/fibrotic zones are replaced by moKCs. In addition to KC death, a LAM-like KC phenotype has been reported in the more fibrotic models of NASH, suggesting that prior to their death, KCs may sense the same signals as the infiltrating LAMs leading to this hybrid phenotype.

(B) LAM-like KCs have also been reported to be present surrounding the damaged zone of the central vein following paracetamol (acetaminophen) overdose. LAMs also develop around the central vein in this model, where these cells potentially interact with activated fibroblasts, necrotic hepatocytes, and other recruited cells, such as neutrophils or cDCs.

steatohepatitis (NASH) (Daemen et al., 2021; Remmerie et al., 2020a; Seidman et al., 2020; Tran et al., 2020), viral hepatitis (Borst et al., 2018), acute liver injury (Ben-Moshe et al., 2022; Graubardt et al., 2017; Kolodziejczyk et al., 2020; Zigmond et al., 2014), bacterial infection (Blériot et al., 2015; Tuin et al., 2018), and hepatocellular carcinoma (HCC) (Lefere et al., 2019) as well as in patients with liver cirrhosis (Ramachandran et al., 2019). However, some studies have used pan-macrophage markers such as F4/80 or CD64 instead of *bona fide* KC markers (Table 2) to reach these conclusions. Moreover, some studies have only assessed KC proportions and not absolute numbers. Thus, whether liver disease always leads to a true reduction in *bona fide* resident KCs remains contentious (Alazawi and Knolle, 2018; Borst et al., 2019a, 2019b; Kessler et al., 2019; Lefere et al., 2019). Further investigation into this is, therefore, needed across diseases utilizing multiple lines of investigation, whereby KC numbers can be accurately assessed. Such studies have recently been completed for NASH (Figure 4), which have identified a significant reduction in resident KC numbers due to their death (Daemen et al., 2021; Remmerie et al., 2020a; Seidman et al., 2020; Tran et al., 2020). KCs have also been proposed to die following infection with *Listeria monocytogenes* (Blériot et al., 2015). Exactly why KCs would be lost across multiple models and whether this would be via conserved or distinct mechanisms remains unclear. By applying the integrated-niche hypothesis, one could postulate that the loss of KCs could be due to the loss of essential cell-cell circuits. Loss of BMP9 production by stellate cells would, for example, lead to loss of resident KCs and the inhibition of moKC development (Guilliams et al., 2022; Zhao et al., 2022). This would also lead to loss of fenestrations in LSECs, blocking hepatic lipoprotein export to circulation (Herrnberger et al., 2014). Such a theoretical model would fit well in NASH, where for example, BMP9 production by stellate cells is abrogated (Yang et al., 2019) possibly due to the trans-differentiation of stellate cells (Hernandez-Gea and Friedman, 2011; Pellicoro et al., 2014). In addition, LSEC fenestrations are lost in fibrosis (Hernandez-Gea and Friedman, 2011; Schaffner and Poper, 1963). Conversely, administration of recombinant BMP9 reduces steatosis, protecting mice from developing NAFLD (Sun et al., 2020); therefore, it is tempting to speculate that this occurs through the re-establishment of BMP9 circuits within the integrated niche. Further fitting with this concept, it has recently been shown that steady-state KCs inhibit stellate cell and hepatocyte activation through their production of miR-690 containing exosomes, but miR-690 production by KCs is lost during NAFLD (Gao et al., 2022). Depletion of miR-690 specifically from KCs results in increased expression of genes associated with inflammation, fibrosis, and lipogenesis, a finding recapitulated upon depletion of KCs or removal of *Rab27* expression from KCs preventing exosome secretion (Gao et al., 2022).

Unlike in homeostatic conditions, resident KCs also fail to proliferate in NASH (Remmerie et al., 2020a; Tran et al., 2020) or following *L. monocytogenes* infection (Blériot et al., 2015). The precise reasons for this are unclear, indeed partial depletion of KCs using *Clec4f-Dtr* mice leads to KC replenishment via two mechanisms, moKC differentiation and KC proliferation (Scott et al., 2016). Moreover, KC proliferation has been observed in acute injury models (Ben-Moshe et al., 2022; Kolodziejczyk et al., 2020; Zigmond et al., 2014), highlighting that this is a mechanism for KC maintenance even in some disease settings. This loss of proliferation in NASH was not due to a loss of proliferative capacity as administration of colony stimulating factor 1-Fc conjugate (CSF1-Fc) triggered KC proliferation (Remmerie et al., 2020a). Perhaps this could suggest that the local concentrations of macrophage growth factors produced by LSECs and stellate cells, such as CSF1 and IL34, are not sufficiently high in

this disease to induce KC proliferation (Guilliams et al., 2020). Another possible explanation for the reduction in resident KCs across models could be that KC loss is a key event regulating downstream hepatic responses to injury, although this does not explain what drives KC loss in the first instance. Consistent with this, KC death has been proposed to be an altruistic mechanism to recruit in effector cells in inflammation (Ginhoux et al., 2017). KC death in the *Clec4f-Dtr* mice leads to TNF-α and IL-1β release from the dying cells resulting in monocyte recruitment to the liver (Bonnardel et al., 2019). Moreover, lipopolysaccharide (LPS) administration to mice leads to a reduction in *Vsig4* expression in total hepatic macrophages coupled with increased *Ly6c2* expression (Tuin et al., 2018). This is coupled with a reduction in plasma CETP concentrations in transgenic mice and humans and an increase in plasma HDL (Tuin et al., 2018), which is proposed to dampen inflammation, as HDL is suggested to play anti-inflammatory roles (Tanaka et al., 2020). Although the precise mode of KC death in the context of many hepatic diseases including NASH remains to be determined, understanding this represents an important question as the mode of death may have considerable consequences for how the liver subsequently responds.

A common theme throughout the literature is that KCs would mediate inflammation and/or resolution by being plastic cells capable of responding to different stimuli. However, we hypothesize that an integrated niche would result in a hardwired epigenome that would greatly limit KC plasticity. As such, we would question whether the plasticity of KCs has been largely overestimated by studying liver macrophages without properly distinguishing *bona fide* KCs from recruited macrophages (Box 1). Indeed, the loss of KCs observed across disease models could also be interpreted as a lack of plasticity, whereby KCs cannot sufficiently adapt to their new environments and, hence, die. This would be consistent with our data from a model of NASH, whereby KCs do not display any activation signal compared with KCs from standard diet fed control mice (Remmerie et al., 2020a). Another possibility would be that KCs are capable of responding initially but that this response cannot be maintained for prolonged periods, ultimately leading to their death. This would be consistent with data from other NASH models and acute liver injury models, whereby remaining KCs or a subset thereof are activated (Ben-Moshe et al., 2022; Kolodziejczyk et al., 2020; Krenkel et al., 2020; Seidman et al., 2020; Tran et al., 2020; Xiong et al., 2019). It would also be consistent with data showing that KCs upregulate their expression of *Il1b*, *Tnf*, *Il10*, and *Il18* 2 hours after administration of LPS *in vivo*, although whether this activation would subsequently lead to KC death remains to be studied (Guilliams et al., 2022). In any case, the question that remains to be answered is what is activating the KCs? Increased lipids have been implicated in driving KC death in NAFLD (Luo et al., 2021; McGettigan et al., 2019), but whether this would also activate KCs remains to be determined.

Notably, in many disease models where KCs are accurately discriminated from recruited macrophages and alterations in the transcriptional programs have been observed, a similar KC activation profile has been reported across models. This profile resembles LAMs (Figure 4), as *Gpnmb*, *Trem2*, *Spp1*, *Cd9*, and *Mmp12* are upregulated but expression of KC genes including *Cd5l*, *Clec4f*, and *Timd4* are maintained (Ben-Moshe et al., 2022; Kolodziejczyk et al., 2020; Seidman et al., 2020; Xiong et al., 2019) (Table 2). Of course, there may also be model-specific KC responses, for example, an inflammatory and endoplasmic reticulum (ER) stress response has been observed in the methionine and choline deficient (MCD) diet model of NASH (Tran et al., 2020). As such, we do not dispute that KCs can become activated in the diseased liver (Zwicker et al., 2021), but rather question whether this represents a profound plasticity of KCs. It will be interesting to dive into the epigenetic profiles of the KC genes that get upregulated during disease. Does this involve usage of *de novo* enhancers that differ from one model to the next or were most of these genes already poised for activation in the steady-state KCs?

Besides investigating how KCs are activated, the relevance of this activation and KC involvement in disease pathogenesis remains to be studied. Recently, KCs have been suggested to be protective in NASH through their role in the clearance of gut-derived microbial DNA containing extracellular vesicles (mEVs) from the bloodstream in a VSIG4- and C3-dependent manner (Luo et al., 2021). Loss of VSIG4 ($CRIg^{-/-}$ mice) results in increased dissemination of mEVs to metabolic tissues leading to exacerbated inflammation and insulin resistance (Luo et al., 2021). An obvious function for KCs as the main phagocytes of the liver is to clear pathogens from the blood. Fitting with such a role, KCs are predominantly found in peri-portal regions of the liver, where the blood enters the liver (Gola et al., 2021; Guilliams et al., 2022). Further highlighting a role for KCs in surveillance, mice with a reduction in KCs are more susceptible to infection with *L. monocytogenes* (Zhao et al., 2022). Intravital imaging studies have also revealed that the ability of KCs to capture and clear bacteria may further contribute to downstream hepatic responses (Kolaczkowska et al., 2015; Wong et al., 2013).

In the context of acute liver injury such as with paracetamol overdose, the ability of KCs to capture and kill circulating bacteria has been reported to be impaired, a finding correlating with increased susceptibility to infection (Triantafyllou et al., 2021). This also correlates with increased expression of PD-1 on KCs during recovery, whereas blocking PD-1 is able to confer protection from bacterial infection (Triantafyllou et al., 2021). This suggests that the ability to clear bacteria can also be modulated in KCs, although how this affects the rest of the cells within the integrated niche is unclear. In this regard, it will be interesting to determine if this is a feature of all KCs or is specific to those in damaged zones. Although in this study, administration of immunotherapy had an advantageous outcome, KCs do not always respond favorably. Rather KCs are, at least in part, responsible for the toxicity observed in the liver following anti-CD40, anti-PD-1, or anti-CTLA4 treatments. This is mediated through their sensing of lymphocyte-derived IFN-γ and their subsequent production of IL-12, which drives a pathogenic response in neutrophils (Siwicki et al., 2021). KCs have also recently been implicated to play a detrimental role in ischemia-reperfusion (I/R) injury, as depletion of KCs utilizing an anti-TIM4 antibody 2 hours before inducing the injury led to a minor protective effect through a currently unidentified mechanism (Ni et al., 2021). Thus, although KC loss appears to be a generic feature of liver disease, the precise functions of KCs in disease appear to be condition specific.

RECRUITED MACROPHAGES IN THE DISEASED LIVER

Liver disease is also associated with the recruitment of monocyte-derived macrophages. These macrophages can acquire distinct phenotypes across, but also within, models (Figure 4). For example, in NASH and viral hepatitis, monocytes entering the liver can differentiate into moKCs (Borst et al., 2018; Daemen et al., 2021; Remmerie et al., 2020a; Seidman et al., 2020; Tran et al., 2020), whereas in other models, such as paracetamol overdose, non-KC macrophages are generated (Ben-Moshe et al., 2022; Zigmond et al., 2014) (Figure 4). These events are not always mutually exclusive, as in NASH, distinct types of monocyte-derived macrophages can develop within the same liver (Daemen et al., 2021; Remmerie et al., 2020a) (Figure 4). The fates of monocyte-derived macrophages across models, however, remain unclear. For example, in *L. monocytogenes* infection, two distinct monocyte-derived macrophage phenotypes have been reported at D1 and D3, respectively; however, whether this is due to a change in phenotype of the recruited macrophages or the presence of distinct populations of recruited cells at the different time points is unclear (Blériot et al., 2015). Similarly, in a murine model of HCC, a population of TIM4$^-$ recruited macrophages has been described, but whether these represent moKCs or another population of monocyte-derived macrophages remains to be determined (Lefere et al., 2019). In the context of human HCC, 3 populations of tumor-associated macrophages (TAMs) have been identified, termed TAM1, 2, and 3, respectively (Sharma et al., 2020). However, further analysis of the TAM1 population has demonstrated considerable homology to KCs (Figure 2). The TAM1 population could also be divided into 2 clusters of which one has been predicted to be monocyte-derived (Sharma et al., 2020), suggesting that HCC may also result in the differentiation of moKCs (part of TAM1s) and other monocyte-derived macrophages (TAM2 and TAM3).

The presence of distinct monocyte-derived macrophage populations during disease leads us to question (1) whether these cells play distinct roles in the diseased liver and (2) whether these macrophage subsets represent distinct cell types or rather activation states. To dissect this, we first need to consider the different populations of monocyte-derived macrophages described to date. moKCs, as described above, acquire many of the hallmarks of resident KCs, although they are typically distinguished at least initially from resident KCs as they lack expression of TIM4 (Remmerie et al., 2020a; Scott et al., 2016). However, as moKCs can acquire TIM4 with time, the only precise way to distinguish between embryonic and moKCs is through the use of chimeras as recently employed by the Gautier lab (Tran et al., 2020). Of course, this does not come without its own caveats, as the irradiation required to generate such chimeras can also influence the integrated niche and, hence, liver function and disease progression (Li et al., 2021; Shimura and Kojima, 2014).

Another fate for monocyte-derived macrophages in hepatic inflammation is to become a LAM (Figure 4). These LAMs, which are homologous to BD-LAMs (Table 2), have originally been reported in NASH, but a similar signature has now also been observed in recruited monocyte-derived macrophages in paracetamol-induced liver injury (Ben-Moshe et al., 2022; Coelho et al., 2020; Daemen et al., 2021; Guilliams et al., 2022; Krenkel et al., 2020; Remmerie et al., 2020a, 2020b; Zigmond et al., 2014). Crucially, this population is not specific to the murine liver, as similar populations have been identified in the obese and cirrhotic human liver (Guilliams et al., 2022; Ramachandran et al., 2019) (Box 1). Indeed, the appearance of LAMs seems to be a shared feature of inflammation across tissues and species (Guilliams et al., 2022; Liao et al., 2020; Ramachandran et al., 2019; Rizzo et al., 2020). Alongside these models where LAMs have been identified, TAM2s in human HCC express *SPP1* and, hence, could resemble LAMs (Sharma et al., 2020). Also, in models of cholangiopathies, monocyte-derived macrophages expand around the bile duct (Guillot et al., 2021), as these macrophages express *Tweak*, another gene specific to LAMs in NASH (Guillot et al., 2021; Remmerie et al., 2020a), it is tempting to speculate that these would also be LAMs. Notably, in addition to being increased in number, in the disease liver, LAMs are also no longer found specifically at the bile ducts but rather can be found at any sites of injury or inflammation (Ben-Moshe et al., 2022; Daemen et al., 2021; Guilliams et al., 2022; Remmerie et al., 2020a) (Figure 4).

At this stage, we do not know whether LAMs represent an endpoint of macrophage differentiation or whether LAMs can become moKCs or vice versa. The immediate precursor for the LAMs, termed C-LAMs expressing CX3CR1, CCR2, and CD63 have been identified (Daemen et al., 2021), but it is unclear how these cells differ from monocytes that will become moKCs. In the *Clec4f-Dtr* model, monocytes entering the liver post KC depletion express LAM markers, which are gradually downregulated over time as the cells become moKCs (Bonnardel et al., 2019; Scott et al., 2016), although whether this is representative of the whole monocyte population en route to becoming moKCs or a subpopulation of LAM-like monocytes that will not become moKCs remains to be seen. Perhaps this differentiation is linear, and the LAM phenotype is a temporary developmental intermediate of monocytes differentiating into macrophages? Perhaps in disease, monocytes are recruited to zones where they do not get the signals to differentiate into KCs and, hence, they remain as LAMs, or they receive completely different signals to become either a moKC or LAM? Future research will require LAM-specific fate-mapping tools to address these questions and gain more insight into this puzzling population. Similarly, a better understanding of the role of the integrated niche and/or phagocytosis in the development of LAMs is required.

What is the role of the different recruited hepatic macrophage populations in the diseased liver? Unfortunately, due to the lack of tools, we still do not have much information regarding this. However, limited evidence gathered to date suggests that recruited cells react differently to injury as compared with resident KCs, again highlighting the importance of distinguishing between these populations. While resident KCs have a slight detrimental activity in I/R injury, the presence of macrophages en route to becoming moKCs further exacerbates injury (Ni et al., 2021). In the context of NASH, moKCs have also been proposed to worsen pathology (Tran et al., 2020). Additionally, replacing KCs with TIM4$^-$ moKCs as mediated through KC-specific loss of *Commd10* also correlates with mildly worse pathology following paracetamol-induced acute liver injury (Cohen et al., 2021).

Based on the fact that LAMs express Osteopontin (encoded by *Spp1*), Osteopontin expression correlates with the disease

stage (Glass et al., 2018), and loss of *Spp1* protects from fibrosis (Arriazu et al., 2017; Honda et al., 2020; Kiefer et al., 2010; Seth et al., 2014), we initially hypothesized that LAMs would be pathogenic in NASH. However, it could also be that these cells are recruited to diseased areas to induce tissue regeneration (Ben-Moshe et al., 2022; Daemen et al., 2021; Remmerie et al., 2020a). As lipids can induce the LAM phenotype *in vitro* (Guilliams et al., 2022), it could be that LAMs and LAM-like KCs are induced following phagocytosis or efferocytosis of damaged or dying cells and that these cells, hence, play a protective role by clearing the tissue damage (Figure 4). This would be consistent with the study from Daemen et al., which has identified LAMs and c-LAMs in crown-like structures around steatotic hepatocytes (Daemen et al., 2021). Here, elimination of CCR2, a receptor required for monocyte efflux from the bone marrow, results in less LAMs in crown-like structures, correlating with exacerbated fibrosis. Of course, elimination of CCR2 will affect all recruited macrophage populations, and it remains to be demonstrated that the exacerbated fibrosis is specifically due to the lack of LAMs. Similarly, Trem2$^{-/-}$ mice are devoid of LAMs in the obese adipose tissue, and this correlates with accelerated weight gain, enhanced body fat accumulation, and hypercholesterolemia (Jaitin et al., 2019), again suggesting a protective function for LAMs. Notably, it remains to be determined if obese Trem2$^{-/-}$ mice are also devoid of hepatic LAMs, and how this may influence NASH progression. In the context of acute and chronic liver injury Trem2$^{-/-}$ mice show impaired resolution of damage (Coelho et al., 2020), suggesting a protective role for LAMs. However, as both LAM-like KCs and LAMs express *Trem2* (Ben-Moshe et al., 2022), further work is required to dissect out the involvement of these distinct cell types. Finally, potentially fitting with a role for LAMs in resolution of tissue damage, in the context of cholangiopathies, monocyte-derived macrophages (putative LAMs) promote *Itgb6* expression in proliferating biliary epithelial cells (BECs), which is critical for repair (Guillot et al., 2021). Thus, taken together, the data generated to date suggest a protective role for LAMs, through their promotion of tissue repair. Although this holds therapeutic promise, as none of these studies have been performed with LAM-specific tools, further studies are required to fully understand the contributions of LAMs to liver repair and disease resolution. As blocking monocyte recruitment with pan-CCR2 inhibitors has been beneficial in preventing fibrosis in phase 1 and 2 clinical trials (Tacke, 2018), LAMs may also be protective or pathogenic at distinct disease stages. Moving forward, it will be crucial to assess LAM fates and functions in both acute and chronic diseases across time points to get a true picture of their involvement during the onset or the resolution phase of distinct liver pathologies.

CONCLUDING REMARKS

It is now clear that KCs are not the only population of macrophages in the steady-state liver. Moreover, in the diseased liver, additional macrophage populations are recruited, including LAMs that are recruited to the diseased zones (Ben-Moshe et al., 2022; Daemen et al., 2021; Remmerie et al., 2020a). This heterogeneity highlights the need for markers to delineate these populations and has prompted the field to rethink the roles previously allocated to KCs when studied with generic macrophage markers (F4/80 or CD68) or tools (clodronate liposomes or *Lyz2-cre* mice). Comparison across species has allowed markers of human and murine KCs to be defined including VSIG4, FOLR2, and *CD5L* (Guilliams et al., 2022). Armed with these markers and the *Clec4f-cre* mouse model enabling KCs to be specifically targeted, we can now begin to reassess KC functions in both health and disease.

Crucially, KCs do not exist in isolation but are embedded in an integrated niche consisting of 4 different cells, which collaborate to enable liver functions. We have highlighted the role of KCs in iron and lipid homeostasis, but the essential role of KCs as scavengers capturing and exporting circulating molecules to hepatocytes will likely apply in more settings. Given their unique location spanning across the sinusoids and the space of Disse (Bonnardel et al., 2019), KCs would also be ideally placed to deliver circulating molecules to hepatocytes by transcytosis, although this remains to be studied. Processes involving multiple cells need to be correctly coordinated, whereby the cell-cell interactions correctly wire the different gene modules. We hypothesize that such an integrated niche would also limit KC plasticity, although more research is required to investigate this and its consequences.

Finally, LAMs have been identified across the healthy and diseased liver, where the precise location of these cells depends on the specific context. The presence of this conserved signature in recruited macrophages and a subset of KCs across disease models raises the question of whether LAMs are a *bona fide* macrophage subset or rather an activation state of macrophages. However, more studies and LAM-specific genetic tools are required to assess this.

In conclusion, although recent studies have shed light on the heterogeneity of hepatic macrophages, this understanding has inevitably raised more questions than it has answered. Having identified the populations, the goal moving forward will be to accurately discriminate between these to understand their unique functional contributions in health and disease and how this is regulated. With this information, will we be able to re-wire hepatic macrophages to obtain specific functional programs to improve liver function and induce liver regeneration benefiting patient health.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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